Comparative Study of the Resveratrol Content of Twenty-one Italian Red Grape Varieties

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The content of resveratrol and some related stilbenes (piceids and piceatannol) in 21 Italian red grape varieties growing in the same edapho-climatic conditions and at the same stage of ripening was measured. Some varieties, like Barbera, Schiava gentile, Corvina and Marzemino, showed a trans-resveratrol content in the skin two to three times higher than the average content of all varieties. Trans- and cis-piceids were found in concentration higher than that of resveratrol, confirming both to be important contributors to the pool of health-promoting molecules present in grapes. Considering the total amount of stilbenes, the highest content was found in Barbera, Franconia, Negroamaro, Corvina and Marzemino, whereas other varieties, such as Montepulciano, Dolcetto, Croatina, Refosco and Primitivo, seem to synthesise very low amounts of these compounds. Barbera grapes also contain a relatively high concentration of trans-piceatannol, a stilbene with even higher antioxidant properties than resveratrol, which means that it could be an interesting variety as a source of health-promoting molecules.

INTRODUCTION

The phytoalexin trans-3,5,4’-trihydroxystilbene (trans-resveratrol) has been reported in grape skins (Creasy & Coffee, 1988; Jeandet *et al*., 1991) and in wines (Siemann & Creasy, 1992; Goldberg *et al*. 1995; Okuda & Yokotsuka, 1996; Ratola *et al*., 2004; Vitrac *et al*., 2005; Naugler *et al*., 2007). The content of stilbenes in the final product depends mainly on the grape variety (Gatto *et al*., 2008), but a variety of other factors, like the climate, soil (Bavaresco *et al*., 2005), canopy management (Bavaresco *et al*., 2008) and the occurrence of pathogen infections can influence the synthesis of these molecules (Bavaresco *et al*., 1997; Romero-Perez *et al*., 2001). Since stilbenes are located mainly in the grape skin, winemaking practices have a great effect on the extraction of these compounds from pomace into the wine (Jeandet *et al*., 1995a; Okuda & Yokotsuka 1996; Vrhovsek *et al*., 1997; Gambuti *et al*., 2004; Atanackovíc *et al*., 2012). For this reason, the content of trans-resveratrol in red wines is much higher than in white ones, although no significant differences have been found in the corresponding grapes (Jeandet *et al*., 1995a; Okuda & Yokotsuka, 1996; Romero-Perez *et al*., 2001).

In the last twenty years there has been a great deal of interest in the presence of trans-resveratrol in wine because of its beneficial effects on human cardiovascular health, derived from its ability to inhibit platelet aggregation (Varache-Lembège *et al*., 2000) and LDL oxidation (Fauconneau *et al*., 1997), and to produce endothelial nitric oxide-dependent vasorelaxation in vivo (Li *et al*., 2000). With regard to cancer, trans-resveratrol inhibits the proliferation of tumour cells and has a cancer-chemopreventive potential, inhibiting cellular events associated with the three major stages of carcinogenesis (Jang *et al*., 1997). However, the concentration of trans-resveratrol is too low to affect the physiology of consumers who consume wine in moderation, and the benefits of the wine consumption on human health therefore are under debate. On the other hand, the physiological effects attributable to the presence of this compound in wine could be affected by the content of other resveratrol derivatives, like cis-resveratrol and resveratrol glucoside isomers, which have some functional properties similar to trans-resveratrol (Chung *et al*., 1992; Jayatilake *et al*., 1993). Cis-resveratrol does not occur naturally in grapes and is generally formed by exposure to UV light (Soleas *et al*., 1995), whereas both the cis- and the trans-isomers of 3-β-glucoside of resveratrol (piceid) have been detected in the grape skin (Waterhouse & Lamuela-Raventos 1994). The contribution of the piceids could be important, as the content of resveratrol glycosides in berry skins is two to four times higher than that of the aglycon forms for the

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cis-and trans-isomers respectively (Romero-Perez *et al*., 2001). In addition, the glycoside derivatives could be more soluble than the aglycones during juice extraction due to their sugar moiety. Hydroxylated resveratrol (piceatannol) was first reported in grapes by Bavaresco *et al*. (2002), and its anticancer properties, through the inhibition of tyrosine kinase and the induction of apoptosis, are well known (Piver *et al*., 2004). Other stilbenes, such as astringin and viniferin, could also contribute significantly to the total stilbene dietary intake from grapes and wine (Vitrac *et al*., 2005).

With the aim of achieving a better assessment of the effect of the grape variety on the content and composition of stilbenes, a comparative study was performed on 21 Italian red grape varieties without fungal diseases growing in the same edapho-climatic conditions and at the same stage of ripening. This work attempts to contribute to enhancing knowledge on the richness of health-related secondary metabolites in Italian grape varieties.

MATERIALS AND METHODS

Grape samples

Grape samples from 21 red cultivars, all of them belonging to *Vitis vinifera* L., were harvested in the same collection-experimental vineyard of 1.2 ha located in Susegana TV, Veneto Region (North-East Italy) in 2010. The vines had the same age (15 years), were grafted onto SO4 rootstock and were planted at a width of 3.0 m between rows and 1.5 m between vines. The vines were Sylvoz pruned with three canes of 10/12 buds each, and the shoots were vertically positioned upwards between three pairs of catch wires. In order to avoid any fungal disease, specific treatments were applied during the season to control grape downy mildew (*Plasmopara viticola*), powdery mildew (*Uncinula necator*) and grey mould (*Botrytis cinerea*). For the latter fungus, three specific fungicides were used after the blooming period. The meteorological data during the ripening period are reported in Table 1.

To minimise the possible ripening effect among berries, the grape samples (about 1000 berries picked randomly from ten plants) were harvested for each variety when the soluble solid content in the vineyard was about 21°Brix. Moreover, once in the laboratory, the grape berries were separated according to their density by flotation in different saline solutions (from 100 to 190 g/L sodium chloride, corresponding to densities of between 1069 and 1125 kg/m^3) (Rolle *et al*., 2011). The comparative study was carried out on berries with a density of 1088 kg/m^3 so that all of them had a similar sugar content of 202 ± 8 g/L (Rolle *et al*., 2011). The sorted berries were washed with water and inspected visually before analysis; those with damaged skins were discarded.

Stilbene extraction

For each sample, a sub-sample of 40 berries was used. The skins were removed manually from frozen berries, then weighed and freeze-dried. One gram of freeze-dried skins was treated as reported by Sun *et al*. (2006), with some modifications. Briefly, the berry skins were introduced into 40 mL of methanol containing 50 μL of hydrochloric acid and 250 μL of internal standard (trans-hydroxyl stilbene, 200 μg/mL in ethanol). After homogenisation for 1 min with Ultraturrax, the samples were maintained for 48 h in closed containers under stirring at room temperature in the dark. The extract containing polyphenols was obtained by centrifugation (5 000 x g, 5 min) and the solid residue was washed twice with 5 mL of methanol. The washing solutions were added to the first supernatant and the mixture was filtered with a 0.2 μm PTFE syringe filter (Advantec, Milano, Italy). After adding 2 mL of water, the extract was almost completely evaporated to dryness under vacuum at 35°C. The dense residue obtained was suspended in 20 mL of water and stilbene compounds were extracted twice for 15 min with 10 mL of ethyl acetate. The upper solvent layer containing stilbenes was recovered carefully. The organic phase was dried by the addition of anhydrous sodium sulphate and filtered through Whatman 589/3 paper. The ethyl acetate fraction was completely evaporated to dryness under vacuum at 35°C. The residue was dissolved in 2 ml of methanol and 50 mM of formic acid (ratio of 1:1) and then centrifuged at 14 000 x g for 10 min. Each grape sample was extracted in duplicate. All organic solvents and acids were obtained from Carlo Erba (Italy).

HPLC analysis

Stilbenes were separated in a C18 Lichrospher column (4 mm x 250 mm, 5 μm, Agilent Technologies, Milano, Italy) using an HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Dual Band UV detector (Waters Corporation, Milford, MA, USA). The mobile phase consisted of 50 mM of formic acid (solvent A) and methanol (solvent B). The gradient program was 0 to 10%B in 3 min, followed by 10 to 30%B in 5 min, 30 to 44%B in 35 min, 44 to 55%B in 2 min, 55 to 75%B in 15 min and 75 to 100%B in 1 min. After washing for 2 min with solvent B, the column was re-equilibrated with solvent A. The flow rate was 1.0 mL/min. The injection volume was 20 µL and the column temperature was set to 40°C. Detection was performed at 306 and 285 nm for trans- and cis-isomers respectively. The concentration of individual stilbenes was quantified at the base of the peak area and calibration curves were derived from the commercially available standards of trans-piceid, trans-piceatannol, trans-resveratrol and trans-

TABLE 1
Meteorological data measured during the ripening period in the experimental vineyard in 2010.

Month	Temperature			Rainfall	Mean relative humidity
	min	max	average	mm	%
JUL	18.43	31.39	24.91	57.40	53.02
AUG	18.65	26.39	22.52	80.60	60.48
SEPT	12.63	23.91	18.27	233.80	64.30

hydroxystilbene between 5 and 40 μg/mL. All the stilbene standards were obtained from Extrasynthese (Genay Cedex, France). Cis-isomers were obtained by exposure of the corresponding trans-molecules to UV light for 1 min and, for their quantification, the extinction coefficient of the trans forms was assumed.

Statistical analysis

Statistical analysis was performed with XLSTAT-Pro 7.1 Software. Data (three replicates for each variety) were analysed with one-way ANOVA and the Tukey multiple comparison test was used to compare the means when significant differences were found in the variance analysis. Linear correlation among variables was measured with Pearson's coefficient.

RESULTS AND DISCUSSION

The extraction of resveratrol and related stilbenes from grape berries is a difficult task, mainly due to their low content. In this case, a protocol for the quantitative extraction of stilbenes from grape skins was used (Sun *et al*., 2006). It included the extraction of total polyphenols with acidified methanol, followed by liquid-liquid extraction with ethyl acetate to separate stilbenes from a large amount of other phenolic compounds. In addition to the protocol of Sun *et al.* (2006), a fixed amount of trans hydroxystilbene was added as internal standard before the extraction procedure. The average recovery of the internal standard, calculated from 18 different extractions, was 96.60 ± 3.93 %. The grapes used in this study were treated in order to avoid any fungal infection and, therefore, to measure only the basal synthesis of stilbenes. In fact, if any infection is present in the bunch, the selection of only healthy berries is not sufficient to guarantee the measurement of the basal level of stilbenes, because, as previously reported (Jeandet *et al*., 1995b), resveratrol is present predominantly in non-infected fruits close to the infected area.

The stilbene content was expressed in μg/g of skins (fresh weight) to better compare the varietal response and to avoid the dilution effect due to different berry sizes. The trans-resveratrol content of different red wine grape varieties showed high variability, ranging from 19 to 508 μg/g (average value of 169 μg/g) in the berry skins (Fig. 1). These results are in the same order of magnitude as those previously published for this stilbene compound. Shi *et al.* (2003) reported an average content of trans-resveratrol of 65.67 μg/g in grape skins. Even selecting clusters in the same vineyard and berries having the same sugar content, replications were sometimes quite different within the same variety, as in the case of Gamay, Teroldego, Lambrusco and Merlot grapes, for example. This confirms the high berry-to-berry variability in resveratrol due to the large number of factors influencing its synthesis.

International varieties (Merlot, Pinot noir, Cabernet Sauvignon and Gamay) showed resveratrol concentrations close to the average content. Merlot grapes, however, presented a higher resveratrol content than Cabernet Sauvignon, which agrees with previous data (Romero-Perez *et al*., 2001). Some Italian autochthonous varieties showed a high concentration of this compound, like Barbera, Schiava gentile, Corvina and Marzemino, whereas others, like Primitivo and Croatina, contain very low amounts. Comparing the resveratrol and anthocyanin contents in berry skins (data not shown), no significant correlation was found, despite the fact that the enzymes involved in their synthesis, stilbene synthase and calchone synthase respectively, can compete for the same substrate, as reported previously (Jeandet *et al*., 1995c).

Cis-resveratrol was detected in the berry skins at very low concentrations (data not shown), and only in grape varieties having a high content of the trans-isomer. This confirms that this molecule is not synthesised in grapes but is the result of the isomerisation of the trans-isoforms promoted by UV light during extraction (Soleas *et al*., 1995).

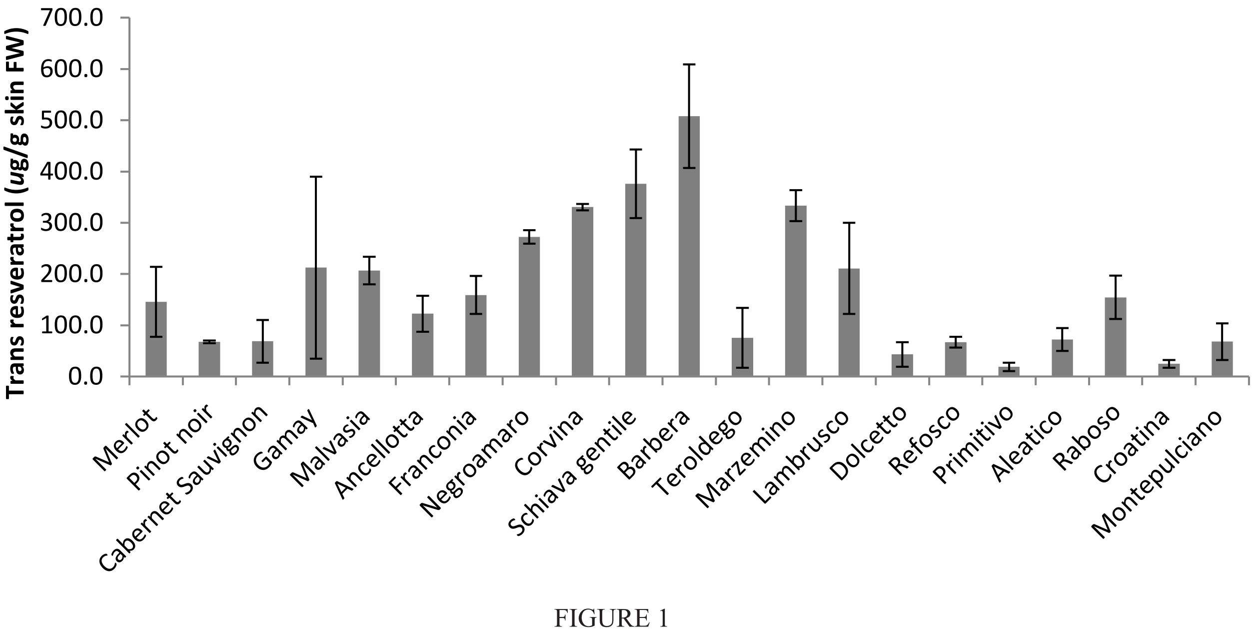

FIGURE 1
Content of trans-resveratrol in skins of 21 different red grape varieties (Lambrusco FF: Lambrusco a foglia frastagliata). Data is expressed as μg/g of fresh skins. Each extraction was performed in duplicate.

The monoglycosides of resveratrol are probably the more represented stilbenes in grapes (Regev-Shoshani *et al*., 2003), and both the cis- and trans-isomers have been identified in grape berries and wines. In the present work, the glycosylated forms of resveratrol were detected in almost all the varieties (except for Montepulciano).

The trans-piceid content in berry skins ranged from not detected to 1 196 μg/g (average value of 260 μg/g) (Fig. 2), in agreement with data previously published showing that the piceid concentration in grapes is generally higher than that of resveratrol (Romero-Perez *et al*., 2001; Sun *et al*., 2006). It can be assumed that the concentration of the glycosylated form is related to that of the corresponding aglycone. However, the correlation coefficient between trans- piceid and trans-resveratrol, even though significant ($p < 0.05$), was only 0.457. In fact, the Franconia variety, with a resveratrol content close to the average value, showed the highest content of trans-piceid. In contrast, Schiava gentile, with one of the highest contents of trans-resveratrol, presented a very low concentration of trans-piceid. This indicates that

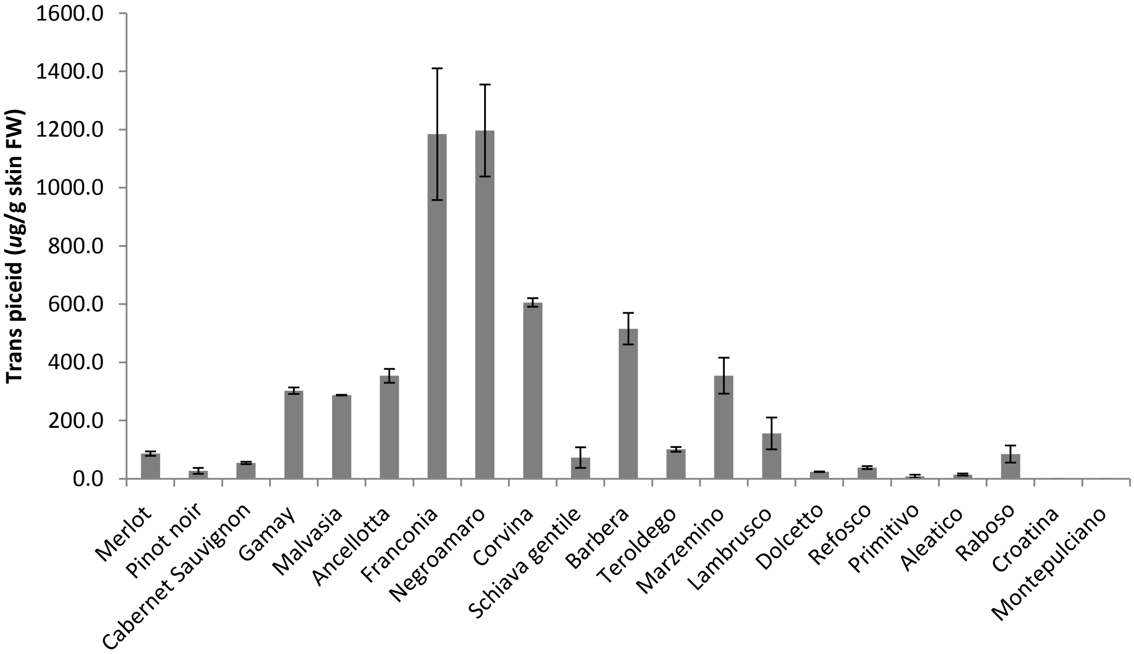

FIGURE 2
Content of trans-piceid in skins of 21 different red grape varieties (Lambrusco FF: Lambrusco a foglia frastagliata). Data is expressed as μg/g of fresh skins. Each extraction was performed in duplicate.

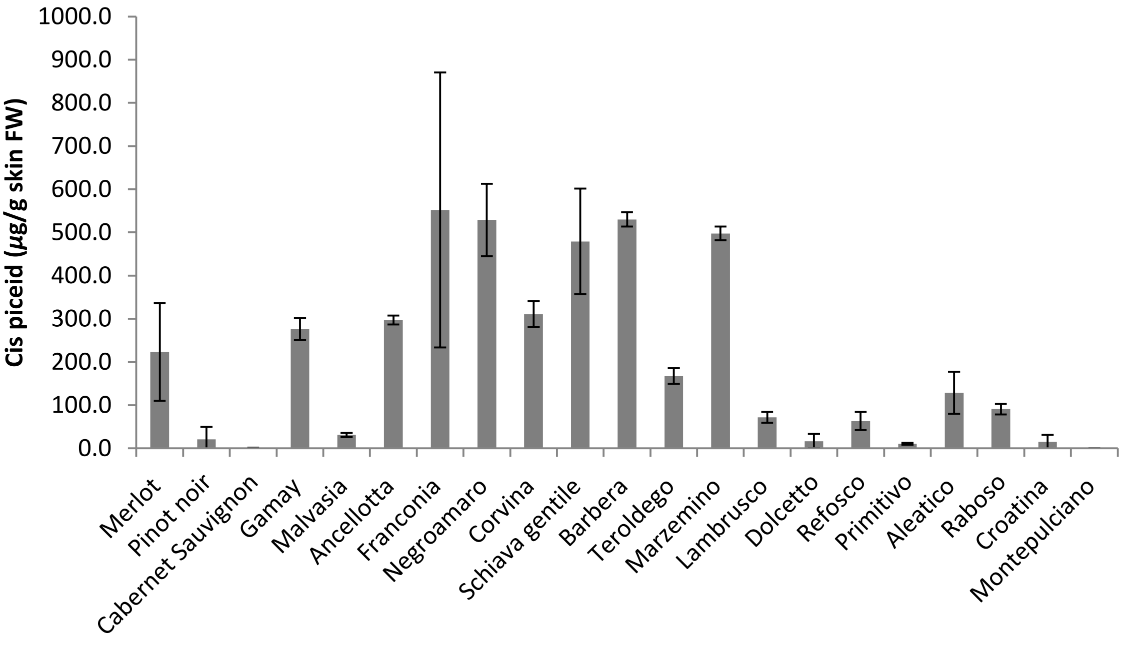

FIGURE 3
Content of cis-piceid in skins of 21 different red grape varieties (Lambrusco FF: Lambrusco a foglia frastagliata). Data is expressed as μg/g of fresh skins. Each extraction was performed in duplicate.

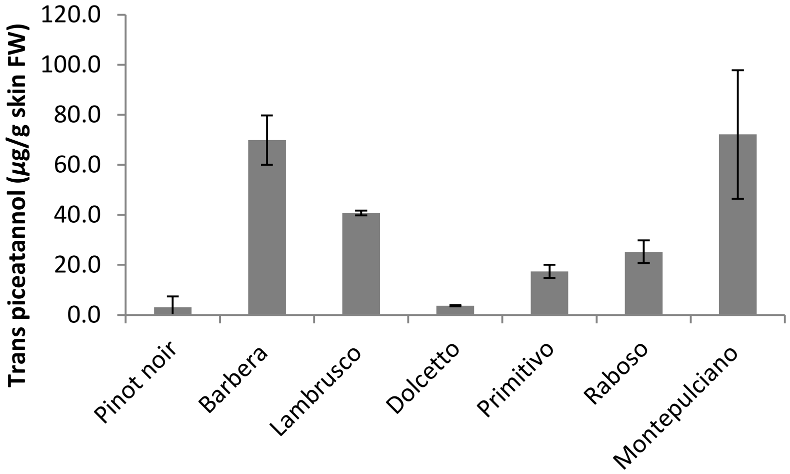

FIGURE 4
Content of trans-piceatannol in skins of 21 different red grape varieties (Lambrusco FF: Lambrusco a foglia frastagliata). Data is expressed as μg/g of fresh skins. Each extraction was performed in duplicate.

the synthesis of the different stilbenes in grape skins could depend on different pathways.

Cis-piceid is also present in high amounts in grape skins (Fig. 3), ranging from not detected to 551 μg/g (average value of 205 μg/g). Both cis- and trans-isomers were previously detected in *V. vinifera* cell cultures, grapes and wines, but the cis/trans ratio was highly variable (Waterhouse & Lamuela-Raventos 1994; Waffo Teguo *et al*., 1998; Ribeiro de Lima *et al*., 1999; Bavaresco & Fregoni, 2001). In the 21 red wine grape varieties studied, the correlation coefficient between the two piceid isomers was 0.736 ($p < 0.05$). Franconia, even though it had large variability between replicates, was still one of the varieties with the highest content of this glycosylated form of resveratrol in berry skins, followed by Negroamaro, Schiava gentile, Barbera and Marzemino. Among international varieties, Merlot showed a higher content of both cis- and trans-glycosides than Cabernet Sauvignon, confirming previous data (Romero-Perez *et al*., 2001).

In some varieties it was also possible to detect a minor stilbene, trans-piceatannol (Fig. 4). Curiously, Montepulciano, which was devoid of both piceid isomers and showed a low content of trans-resveratrol, was the variety with the highest mean content of piceatannol (72.1 μg/g). On the other hand, Barbera, which contained large quantities of resveratrol and piceids, also showed a high content of piceatannol (69.9 μg/g). Even at low concentrations, piceatannol can play an important role in contributing to health properties, because the additional hydroxyphenyl group in the B ring (catechol structure) significantly increases the trapping effect of free radicals and antioxidative properties. Piceatannol doses of 25 μg/kg show antiarrhythmic and cardioprotective properties in rats (Hung *et al*., 2001).

CONCLUSIONS

In the survey of 21 red wine grape varieties, including most of the autochthonous Italian cultivars, a large variability was found in stilbene content. Some varieties, such as Barbera, Franconia, Negroamaro, Corvina and Marzemino, showed a very high content of both resveratrol and its monoglycosides in the berry skins. In particular, Barbera grapes, which also contain a relatively high concentration of trans-piceatannol, could be an interesting variety as a source of health-promoting molecules. On the other hand, other varieties, such as Montepulciano, Dolcetto, Croatina, Refosco and Primitivo, seem to synthesise very small amounts of stilbenes.

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